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Effects of plant-litter quality on litter decomposition by benthic invertebrates along a gradient of eutrophication

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Abstract

Human impacts on freshwater ecosystems, such as nutrient enrichment and changes in riparian vegetation, are increasing, and policy makers are making efforts to identify management priorities. A critical point is the assessment of the ecological integrity of ecosystems. Benthic invertebrate communities have been widely used to assess the structural condition of streams, because they integrate the effects of stressors through time. However, structural measures do not provide information on ecosystem function making it difficult to actually assess the ecological condition of a stream.

In streams, leaf-litter decomposition is a key ecosystem process that links riparian vegetation, physico-chemical environment and decomposer communities. In this study, we assessed the effects of litter quality and eutrophication on plant-litter decomposition and benthic invertebrates in streams. Leaves of five plant species with different chemical composition, namely *Alnus glutinosa* (alder), *Castanea sativa* (chestnut), *Eucalyptus globulus* (eucalyptus), *Platanus* sp. (plane tree) and *Quercus robur* (oak), were enclosed in coarse-mesh bags and immersed during 37 days in 7 streams of Northwest Portugal, along a gradient of eutrophication.

Overall, the structure of benthic macroinvertebrate communities was sensitive to the water quality degradation and was able to discriminate the less eutrophic from the most eutrophic streams. Leaf-litter decomposition was stimulated until intermediate levels of eutrophication and decreased in the most eutrophic streams, where the high concentration of nutrients and the possible occurrence of pollutants could have inhibited decomposition. Leaf mass loss of alder leaves was significantly faster when compared to other leaf species, and macroinvertebrate community showed evidence of preferential colonization of high quality leaves.

In conclusion, leaf decomposition showed to be a promising tool to assess changes in stream water quality. In addition, comparing to macroinvertebrate community analysis, estimation of leaf mass loss has advantages associated with costs, time of operation and simplicity of application. Overall, results from this study showed that both structural and functional measures were good predictors of eutrophication in streams. Therefore, these measures complemented each other and make an integrative assessment of the ecological condition of streams possible.

Resumo

Os impactos humanos nos ecossistemas de água doce, como a eutrofização e as alterações na vegetação ripária, estão a aumentar, e os decisores políticos estão a fazer esforços para identificar as prioridades de gestão. Um ponto crítico é a avaliação da integridade ecológica dos ecossistemas. As comunidades de invertebrados bentónicos têm sido amplamente usadas para avaliar a condição estrutural dos rios, porque elas integram os efeitos dos stressores ao longo do tempo. Contudo, as medidas estruturais não providenciam informação sobre o funcionamento do ecossistema tornando difícil a avaliação da condição ecológica dos rios.

Nos rios, a decomposição da folhada é um processo chave do ecossistema que depende da vegetação ripária, do ambiente físico-químico e das comunidades decompositoras. Neste estudo, foram avaliados os efeitos da qualidade das folhas e da eutrofização na decomposição da folhada e nos invertebrados bentónicos em 7 rios do Noroeste de Portugal. Folhas de cinco espécies de plantas, com diferente composição química, nomeadamente *Alnus glutinosa* (amieiro), *Castanea sativa* (castanheiro), *Eucalyptus globulus* (eucalipto), *Platanus* sp. (plátano) e *Quercus robur* (carvalho), foram colocadas em sacos de malha grossa e imersas nos rios, durante 37 dias, ao longo de um gradiente de eutrofização.

Em geral, a estrutura da comunidade de macroinvertebrados bentónicos foi sensível à degradação da qualidade da água e foi capaz de discriminar os rios segundo o grau de eutrofização. A decomposição da folhada foi estimulada até níveis intermédios de eutrofização e diminuiu nos rios mais eutrofizados, onde as concentrações de nutrientes e a possível co-ocorrência de outros poluentes podem ter inibido a decomposição. A perda de massa das folhas de amieiro foi mais rápida quando comparada com as outras espécies de folhas, e a comunidade de macroinvertebrados colonizou preferencialmente as folhas de qualidade elevada.

Em conclusão, a decomposição da folhada mostrou ser uma ferramenta promissora para avaliar alterações na qualidade da água dos rios. Além disso, comparando as metodologias de análise da comunidade de macroinvertebrados com as usadas para determinar a perda de massa das folhas podemos constatar que esta última tem vantagens associadas com os custos, o tempo de execução e a simplicidade de aplicação. Globalmente, os resultados deste estudo mostraram que as medidas estruturais e funcionais foram bons indicadores do nível de eutrofização dos rios. Por conseguinte, estas medidas são complementares, tornando possível uma avaliação integrativa da condição ecológica dos rios.

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1. Introduction

1.1. Plant-litter decomposition in freshwater ecosystems

Streams have two possible sources of primary energy: instream photosynthesis carried out by algae, mosses and higher aquatic plants, and the organic matter imported from the riparian vegetation, which falls in the stream channel (Benfield 1996). In low-order forest streams, light availability is generally reduced by the surrounding canopy trees, that limit instream photosynthesis; so, energy supplies is largely supported by the imported organic matter (Benfield 1996, Abelho 2001), that contributes in 99% to the total energy input (Fisher and Likens 1973).

The allochthonous organic matter is mainly composed by leaves, branches, seeds, fruits, flowers and other plant parts from the riparian vegetation (Cummins 1974), which can be trapped in the stream reach and become available for aquatic biota or be transported downstream (Elosegi 2005). Leaves that enter into the stream are usually the principal energy resource for aquatic biota (Abelho 2001). Indeed, Abelho and Graça (1996) found that the contribution of leaves from a deciduous forest could attain 72%, whereas the contribution of wood and reproductive plant material was only 16% and 12%, respectively.

When leaves fall into streams three main stages can be considered during litter breakdown: leaching, conditioning and fragmentation (for a review see Webster and Benfield 1986, Gessner et al. 1999, Abelho 2001). Although these stages of leaf decomposition tend to occur sequentially in time, some of them can occur simultaneously (Gessner et al. 1999, Allan and Castillo 2007). Leaching is the process that occurs mainly at first stages of leaf breakdown and consists in the rapid loss of soluble compounds (up to 30%), such as polyphenolics, carbohydrates and amino acids, in the first 24 hours to 7 days after leaf immersion (Petersen and Cummins 1974, Webster and Benfield 1986, Canhoto and Graça 1996, Casas and Gessner 1999). Conditioning is the colonization of plant litter by aquatic microorganisms, mainly fungi and

bacteria, which degrade plant litter and enhance leaf palatability to invertebrate shredders (Cummins 1974, Suberkropp 1998, Graça 2001). Significant microbial colonization occurs within one or two weeks of leaf immersion (Cummins 1974). Fragmentation results from the abrasion and shear stress exerted by the flowing water (Gessner et al. 1999), and also from biotic fragmentation due to the feeding and digestive activities of shredders, which contribute to the release of fine particulate organic matter (FPOM) (Cummins 1974, Allan and Castillo 2007). At the end, the original plant material, constituted by coarse particulate organic matter (CPOM) is transformed into several products, including biomass of shredders, fungi and bacteria, dissolved organic matter (DOM), FPOM, inorganic nutrients and carbon dioxide (Gessner et al. 1999).

In streams, leaf breakdown is controlled by several factors, such as water temperature (Rowe et al. 1996, Fernandes et al. 2009), pH and alkalinity (Dangles et al. 2004), concentration in nutrients (e.g., Pascoal et al. 2001, 2003, Gulis and Suberkropp 2003a, b), climate and hydrologic fluctuations (for a review see Gessner et al. 1999, Abelho 2001) and chemical and physical characteristics of leaves (Petersen and Cummins 1974, Schindler and Gessner 2009).

1.2. The role of aquatic biota in plant-litter decomposition

Plant-litter decomposition is a key process in aquatic ecosystems that involves the activity of three groups of organisms: fungi, bacteria and invertebrate detritivores (Suberkropp 1998, Gessner et al. 1999, Graça 2001, Hieber and Gessner 2002, Pascoal et al. 2005a).

Fungi, mainly aquatic hyphomycetes, also known as Ingoldian fungi, are the main colonizers of the CPOM that enters into streams (Cummins and Klug 1979, Bärlocher 1992). Aquatic hyphomycetes are well adapted to the stream environment (Bärlocher 1992), due to their morphological and physiological adaptations that allow them to colonize and use plant litter in flowing waters

(Suberkropp 1998). Morphological adaptations include tetraradiate and sigmoid shapes of asexual spores and the production of mucilages at the end of the spore arms (Read et al. 1992) that facilitate the attachment to the substrates they colonize (Suberkropp 1998). Physiological adaptations include the ability of producing a variety of extracellular enzymes that degrade the major structural plant cell-wall polysaccharides, like cellulose, pectin and xylan (Suberkropp and Klug 1980, Chamier 1985, Suberkropp 1992). Aquatic hyphomycetes are able to quickly colonize a wide range of substrates, such as leaves and wood, to grow and rapidly produce spores, and to complete their life cycle within few weeks (Bärlocher 2005, Gessner et al. 2007).

Bacteria are also capable of producing enzymes that degrade the polysaccharides of plant litter (Burns 1982), but its contribution to plant-litter decomposition is lower than that of fungi (Gessner and Chauvet 1994, Baldy et al. 1995, 2002, Hieber and Gessner 2002, Gulis and Suberkropp 2003a, b, Pascoal and Cássio 2004, Pascoal et al. 2005a). The lower contribution of bacteria can be related to the lack of invasive ability, which limits bacterial cells to the leaf surface (Porter et al. 1989, Suberkropp and Klug 1974). Because of that, bacteria increase its role in leaf decomposition only after leaf material has been partially broken down (Baldy et al. 1995, 2002, Pascoal and Cássio 2004). However, in polluted rivers with depressed fungal activity, bacteria can increase its relative contribution to leaf-litter decomposition (Pascoal and Cássio 2004).

Shredders and collectors comprise the invertebrates that feed on CPOM and FPOM, respectively (Graça 2001, Allan and Castillo 2007). Since shredders have mouthparts adapted for the maceration of CPOM, they have a major role in litter breakdown (Allan and Castillo 2007). The result of its feeding activity is the conversion of CPOM into FPOM, with the production of a large quantity of fecal pellets, that constitute an important food source for deposit feeders and filter feeders (Graça 2001, Canhoto and Graça 2006).

Several studies have shown that shredders feed preferentially on conditioned plant detritus (Graça et al. 1993, Graça et al. 2001, Graça and

Cressa 2010), because the colonization by microbes, especially aquatic hyphomycetes, promotes plant tissue softening (Bärlocher and Kendrick 1975, Graça et al. 1993) and provides mycelia as food for shredders (Arsuffi and Suberkropp 1988, Graça et al. 1993). Besides, invertebrates may selectively feed on leaves colonized by particular fungal species (e.g., Bärlocher and Kendrick 1973, Arsuffi and Suberkropp 1985, Graça et al. 1993, Lecerf et al. 2005). Several studies have shown that shredder invertebrates contribute significantly to litter breakdown (e.g., Robinson and Gessner 2000, Hieber and Gessner 2002, Lecerf et al. 2005). For example, the exclusion of shredders of a stream by application of an insecticide led to a decrease in leaf breakdown rates (Wallace et al. 1996).

1.3. Effects of litter quality on plant-litter decomposition and benthic macroinvertebrates

Leaf litter from riparian vegetation that falls into streams is an abundant food resource for aquatic food webs (Petersen and Cummins 1974, Vannote et al. 1980). Riparian corridors can be composed by a high diversity of tree species with different leaf traits, such as toughness, nitrogen, polyphenol and lignin contents that have potential to affect litter decomposition rates (for a review see Gartner and Cardon 2004) and stream biota (Abelho and Graça 1996, Sampaio et al. 2001, Lecerf et al. 2005).

Leaf decomposition is fast for leaf species with high concentrations of nutrients, such as nitrogen and phosphorus, and slow for leaf species with high concentration of recalcitrant or inhibitory compounds, such as lignin (Schindler and Gessner 2009) or polyphenols (Bärlocher et al. 1995, Canhoto and Graça 1999). For instance, high decomposition rates of alder leaves have been associated with high nitrogen and phosphorus content in leaves (Pozo et al. 1998, Sampaio et al. 2001), while the lower decomposition rates of eucalyptus leaves (Pozo et al. 1998, Sampaio et al. 2001) are probably due to lower content in nitrogen and phosphorus, higher polyphenolic content, and

the presence of a waxy cuticle that acts as a physical barrier to microbial colonization (Bärlocher et al. 1995, Canhoto and Graça 1999) and macroinvertebrate feeding (Canhoto and Graça 1999).

Some experiments have shown that shredder invertebrates colonize preferentially high quality leaves, such as alder (e.g., Irons et al. 1988, Pozo et al. 1998, Sampaio et al. 2001, Lecerf et al. 2005), rather than low quality leaves, such as oak and/or eucalyptus (Pozo et al. 1998, Sampaio et al. 2001, Lecerf et al. 2005). However, Sampaio et al. (2001) observed that shredder density increased in low quality leaves after leaching of the recalcitrant compounds. Laboratory studies have shown that the shredders *Sericostoma vitatum* and *Tipula lateralis* reject eucalyptus leaves over other more labile leaf species (Canhoto and Graça 1995). Other manipulative experiments have shown that the removal of oils from eucalyptus leaves increased leaf consumption by *T. lateralis* (Canhoto and Graça 1999). On the contrary, the addition of eucalyptus-extracted polyphenols and oils to alder leaves reduced invertebrate consumption (Canhoto and Graça 1999).

So, given the evidence that leaves that fall from the riparian vegetation is the major energy resource for aquatic communities, shifts in riparian tree species composition is expected to alter the quantity and quality of litter input into streams with effects on the structure and activity of aquatic communities (Kominoski et al. 2009).

1.4. Effects of eutrophication on freshwater ecosystems

Eutrophication caused by anthropogenic activities can affect directly stream biota and ecosystem functioning, through the increase of nutrient concentrations in stream water, mainly phosphorus and nitrogen (Vitousek et al. 1997, Carpenter et al. 1998). Generally, the increase of dissolved inorganic nutrients in stream water is reported to accelerate plant-litter decomposition (Pascoal et al. 2003, 2005a, Gulis and Suberkropp 2003a, b, Ferreira et al. 2006, Castela et al. 2008, Menéndez et al. 2011). However,

other studies have shown no effect of phosphorus on litter decomposition in headwater streams (Lecerf et al. 2006, Baldy et al. 2007) or reported a decrease in leaf breakdown rates along an eutrophication gradient, defined by ammonium concentrations in stream water (Lecerf et al. 2006). Furthermore, in some nutrient-enriched sites with low oxygen concentration and sedimentation, leaf breakdown can be depressed suggesting that the positive effects of nutrients can be reduced by other factors (Pascoal and Cássio 2004, Pascoal et al. 2005a, Mesquita et al. 2007).

Inorganic nutrients are also reported to stimulate fungal (Pascoal et al. 2003, 2005a, Ferreira et al. 2006, Suberkropp et al. 2010, Menéndez et al. 2011) and bacterial (Gulis and Suberkropp 2003a, b, Pascoal and Cássio 2004, Baldy et al. 2007, Suberkropp et al. 2010) activity, and to increase the abundance (Robinson and Gessner 2000, Pascoal et al. 2001, 2003, Gulis et al. 2006) and biomass (Robinson and Gessner 2000, Greenwood et al. 2007), but not richness (Pascoal et al. 2001, 2003, Bergfur et al. 2007b) of invertebrates on decomposing leaves. On the other hand, Ferreira et al. (2006) found that the abundance and richness of leaf-associated invertebrates did not respond to the addition of nitrates in stream water till concentrations of 983 µg/L. Although invertebrate biomass and abundance can be stimulated by moderate eutrophication, in hypertrophic streams, shredder biomass can decrease drastically (Baldy et al. 2007). The increase of nutrients has also been reported to alter the community structure of fungi (Gulis and Suberkropp 2004, Pascoal et al. 2005b, Castela et al. 2008) and invertebrates (Pascoal et al. 2001, Castela et al. 2008).

1.5. Evaluation of ecological integrity of freshwater ecosystems

Streams and rivers provide numerous benefits to human civilization, such as water purification, routes for travel and transport, renewable energy, water supply for industrial, domestic and agricultural uses, recreation and harvestable organisms (Allan and Flecker 1993, Allan and Castillo 2007).

However, due to the influence of human activities, aquatic ecosystems become highly stressed and dysfunctional (Vitousek et al. 1997).

Some authors have reviewed the current threats to rivers (Allan and Flecker 1993, Malmqvist and Rundle 2002, Dudgeon et al. 2006, Strayer 2006). The threats imposed on freshwater biodiversity and ecosystems are diverse and can be grouped into five interacting categories: overexploitation, water pollution, flow modification, destruction or degradation of habitats, and invasion by exotic species (for a review see Malmqvist and Rundle 2002, Dudgeon et al. 2006, Vörösmarty et al. 2010).

Concern about the degradation of water resources has been increasing, leading many industrialized countries to seek for measures to improve and restore the water quality and the ecological integrity of streams; as a consequence, a vast array of biological monitoring approaches have been developed (Bunn and Davies 2000, Gessner and Chauvet 2002). According to Karr and Dudley (1981), the concept of ecological (or biological) integrity can be expressed as the "ability of an aquatic ecosystem to support and maintain a balanced, integrated, adaptive community of organisms having a species composition, a diversity, and a functional organization comparable to that of natural habitat of the region". Ecological integrity can be divided into both structural and functional integrity (Gessner and Chauvet 2002). The structural integrity can be defined as the qualitative and quantitative composition of communities and their resources, while the functional integrity refers to the rates, patterns and relative importance of different ecosystem-level processes (Gessner and Chauvet 2002).

In the past, for water management purposes, the evaluation of human impacts on freshwater ecosystems was based exclusively on physical and chemical characteristics of stream water (Karr 1991). However, the recognition that physical and chemical data are not sufficient to assess water quality, led to the inclusion of biological parameters in monitoring programs (Karr 1991). In fact, assessing the ecological condition or the ecological status of freshwater ecosystems has been an important management issue

worldwide. For instance, European countries seek through the Water Framework Directive (WFD, 2000/60 EU) the development of a community action plan, with the aim of ensuring the management and protection of freshwater resources, in order to avoid the deterioration of their quality and quantity (Council of the European Communities 2000). The USA, through the 1972 amendments of the federal Clean Water Act, wants to “restore and maintain the chemical, physical and biological integrity of the nation’s waters” (Mebane 2001). Australia has also developed a national program, the Ausrivas (Marchant et al. 1997), based on the application of a multivariate model that uses undisturbed reference sites to predict which species have high probability of occurring at a site based on environmental measurements that characterize that site (Marchant et al. 1997). In South Africa, it has also been developed a national monitoring program that incorporates several groups of biota for ecological assessment (Roux et al. 1999).

At the moment, the ecological integrity of freshwater ecosystems is being assessed exclusively based on structural aspects of aquatic communities (Barbour et al. 1999, EU 2000). Benthic macroinvertebrates, due to their differential sensitivity to environmental changes and their wide geographical distribution, have been used as biological indicators of freshwater ecosystem health (Rosenberg and Resh 1993), through the application of diversity metrics (Barbour et al. 1999) and biotic indexes (Ferreira et al. 2004). Other biological communities, such as fishes (Magalhães et al. 2008), diatoms (Resende et al. 2010) and macrophytes (Ferreira et al. 2005) have also been used as bioindicators. However, to assess the ecological integrity of freshwaters, both structural and functional components should be considered (Pascoal et al. 2001, Gessner and Chauvet 2002, Pascoal et al. 2003). Plant-litter decomposition is a key ecosystem process that responds to water quality (Pascoal et al. 2001); therefore, it was proposed as a functional measure to assess ecosystem integrity of freshwaters (Gessner and Chauvet 2002, Pascoal et al. 2003). Some studies have shown that litter breakdown is useful to access

eutrophication in streams (Pascoal et al. 2001, Pascoal et al. 2003, Gulis et al. 2006, Lecerf et al. 2006), while others have concluded that this is not always the case (Hagen et al. 2006). Some authors found that litter-breakdown rates may not vary significantly along a gradient of land use because of the confounding effects that agricultural land use have on breakdown rates: positive effects of nutrients offset by the negative effects of sedimentation (Niyogi et al. 2003, Hagen et al. 2006).

Most studies have used *Alnus glutinosa* (L.) Gaertner (alder) to follow leaf decomposition in streams with different levels of human impacts (Pascoal et al. 2001, 2003, Lecerf et al. 2006, Bergfur et al. 2007a). However, because alder leaves are more susceptible to mechanical fragmentation and shredder fedding, it was sugested the use of a tough, low nutrient and slow decomposing leaves, such as oak (Gulis et al. 2006), for assesing the ecological integrity of streams. Mesquita et al. (2007) found that breakdown rates of eucalyptus leaves and the associated decomposer communitie are also sensitive to changes in water quality and can be useful for assessing stream integrity. Nevertheless, more studies on how diferent litter quality responds to different levels of water quality degradation are needed if we want to predict wich leaf species is the most valuabe for assessing stream functional integrity.

1.6. Aim and outline of the thesis

To better understand the impacts of anthropogenic activities on freshwater ecosystems, the effects of eutrophication and the quality of riparian vegetation on plant-litter decomposition and benthic macroinvertebrates were investigated. For that, leaves of five common plant species with different chemical composition, namely alder, chestnut, eucalyptus, plane tree and oak, were enclosed in coarse-mesh bags and immersed in seven low-order streams of Northwest Portugal, along a gradient of eutrophication. After 37 days of leaf immersion, leaf mass loss, the content

of leaf carbon and nitrogen, and the leaf-associated macroinvertebrate communities were examined. Benthic macroinvertebrate samples were also taken from each site, by kicking and sweeping all available biotopes in proportion to their occurrence. Benthic macroinvertebrate derived metrics and biotic indices, namely the Iberian Biological Monitoring Working Party (IBMWP) and the Shannon diversity index, Pielou eveness index, invertebrate abundance, taxon richness, %EPT taxa, and %Oligochaeta taxa, were used as a measure of structural integrity. Leaf-litter decomposition was used as a measure of functional integrity, and results between five plant species were compared to evaluate which species was more reliable for assessing stream integrity.

2. Materials and methods

2.1. Sampling sites

Field studies were carried out at seven sites in streams of the Ave River basin, located in Northwest of Portugal, where granitic rocks dominate the geological substratum. Three sampling sites, Costa Stream, Couros Stream and Selho River, are located near the city of Guimarães in an area with high population density, and agricultural and industrial activities. Other three sites, Andorinhas Stream, Oliveira Stream and Agrela Stream, are located nearby small human settlements with some agricultural activities, while the last sampling site, Agra Stream, is located in the Cabreira Mountain, in an area without apparent human impacts (Fig. 2.1). Elevation of all sampling sites ranged from 778 (Agra Stream) to 149 meters (Selho River and Couros Stream) (Table 2.1). At the sampling sites, Costa Stream is a 2nd order stream, Agra Stream, Oliveira Stream, Andorinhas Stream and Agrela Stream are 3rd order streams, while Selho River and Couros Stream are 4th order streams. All sampling sites were surrounded by a riparian corridor composed by native deciduous trees, namely alder (*Alnus glutinosa* Gaertn.), poplar

(*Populus nigra* L.), chestnut (*Castanea sativa* Miller) and oak (*Quercus* robur L.). In Agrela Stream site, besides the native trees, the exotic eucalyptus (*Eucalyptus globulus* Labill.) was also present in the riparian vegetation. The channel substratum was very diverse at all sites (Table 2.1).

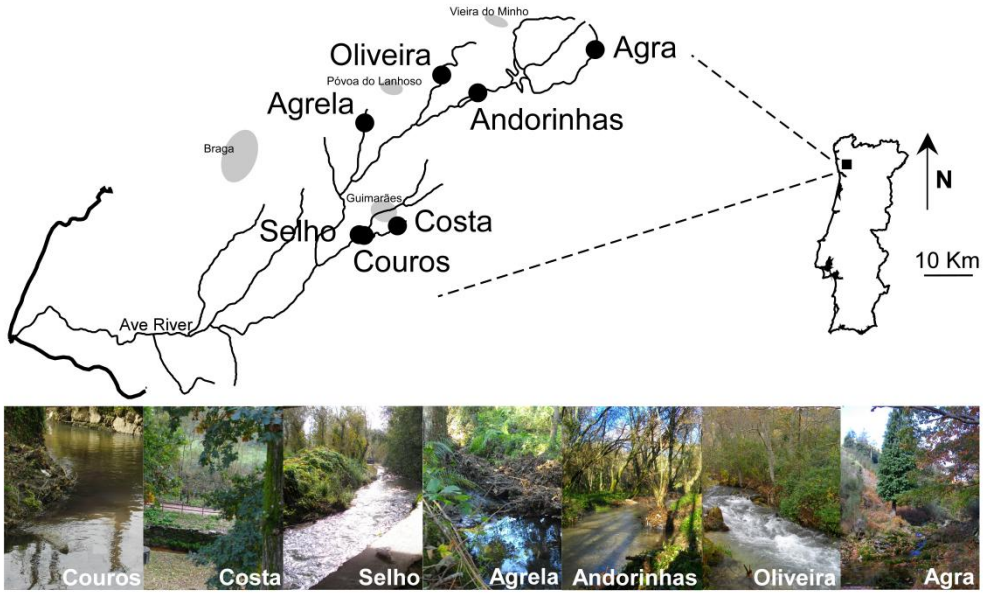

Figure 2.1. Location of the sampling sites and main towns along the Ave River basin, Northwest Portugal.

2.2. Physical and chemical analyses of the stream water

At each sampling site, physical and chemical parameters of stream water were periodically measured during the study period. Temperature, pH, dissolved oxygen and conductivity of the stream water were measured *in situ* with field probes (Multiline F/set 3 no. 400327, WTW). Stream water samples were collected into plastic bottles, transported in a cool box (4ºC), and used within 24h for chemical analyses. Concentrations of nitrite (HACH kit, program 371), nitrate (HACH kit, program 351), phosphate (HACH kit, program 490) and ammonia (HACH kit, program 385) were determined using a HACH DR/2000 spectrophotometer (Hach Company, Loveland, CO, USA).

2.3. Experimental setup

The experimental design included five leaf species with different chemical composition, selected according to their occurrence in the riparian zones of the study area. The selected leaf species were: *Alnus glutinosa* (alder), *Castanea sativa* (chestnut), *Eucalyptus globulus* (eucalyptus), *Platanus* sp. (plane tree) and *Quercus robur* (oak). Leaves were collected immediately before abscission in autumn 2009, air dried and stored until used. Leaves of each plant species were weighed into 4g groups, placed in plastic coarse-mesh bags (5mm mesh size; 23x30cm) to allow invertebrate colonization. Pebbles were placed into leaf bags to maintain them on the stream bottom. The bags were sealed, tied to riparian trees and immersed at each sampling site on 10 November 2010. After 37 days, bags were retrieved from the streams, placed individually in plastic bags and transported to the laboratory. In the laboratory, samples were washed with tap water through a battery of sieves to remove sand, exogenous organic matter and macroinvertebrates. Invertebrates retained on the sieves were sorted and preserved in 96% ethanol. All macroinvertebrates were counted and identified, under a stereo microscope (Leica Zoom 2000), to the lowest possible taxonomical level (Tachet et al. 2010). Invertebrates were assigned or not to shredders according to Merritt and Cummins (1996).

2.4. Leaf dry mass

Leaf species from each replicate bag were freeze-dried to constant mass (72±24h) and weighed (±0.001g). Four additional bags of each leaf species were done but kept in the laboratory as control to determine initial leaf freeze-dried mass.

Table 2.1. Location, physical characteristics and composition of riparian vegetation at the sampling sites in the Ave River basin.

Sampling site	Longitude N	Latitude W	Elevation (m)	Stream order	Width (m)	Riparian vegetation	Substratum type
Agra Stream	41º36'35.38''	8º02'19.89''	778	3	0.3-0.6	*Quercus robur, Castanea sativa*	rock, boulder, gravel, vegetation
Oliveira Stream	41º35'10.92''	8º13'30.84''	251	3	0.2-0.4	*Alnus glutinosa, Quercus robur, Platanus hispanica, Castanea sativa*	rock, boulder, gravel, sand, vegetation
Andorinhas Stream	41º34´13.39''	8º10'35.71''	200	3	0.3-1	*Alnus glutinosa, Quercus robur, Castanea sativa*	rock, gravel, sand
Agrela Stream	41º32'30.45''	8º19'09.96''	325	3	0.6-1	*Alnus glutinosa, Quercus robur, Eucalyptus globulus*	rock, sand, silt
Selho River	41º26'17.94''	8º19'21.49''	149	4	0.7-1	*Alnus glutinosa, Populus nigra*	rock, sand, silt
Costa Stream	41º26'53.84''	8º16'34.89''	226	2	0.4-0.5	*Alnus glutinosa, Quercus robur, Populus nigra, Castanea sativa*	rock, gravel, sand
Couros Stream	41º26'15.02''	8º19'19.24''	149	4	0.5	*Populus nigra*	gravel, sand, silt

2.5. Nitrogen and carbon content of leaf litter

Nitrogen and carbon content was determined from samples of each leaf species (±120mg) before and after leaf immersion in the streams. The analyses were done with a LECO-CNS 2000 Analyzer (Leco Corp., St. Joseph, MI, USA) at the Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI) in Spain.

2.6. Macroinvertebrate sampling

At each sampling site, benthic macroinvertebrates were harvested during the late autumn and early winter of 2010/2011, with a hand net (60x30cm; 0.5mm mesh size), by kicking and sweeping all available habitats according to its proportion. Additionally, stones and submerged vegetation were also examined to collect invertebrates. Samples were transferred to zip plastic bags, brought to the laboratory in a closed bucket and stored at 4ºC (<48 hours) until sorting. In the laboratory, each sample was washed with tap water through a battery of sieves, and macroinvertebrates were sorted and preserved in 96% ethanol for further identification, as described above.

2.7. Data analyses

Ordination of the streams according to the stream water variables was done using a Principal Component Analyses (PCA), after standardization of data (Legendre and Legendre 1998).

Two-way analyses of variance (two-way ANOVA) were used to test if leaf mass loss or leaf-associated invertebrates depended on plant-leaf species and the stream, followed by Tukey's HSD tests (Zar 1996). Because data of leaf mass loss had a normal distribution, no data transformation was done. Invertebrate data were log(x+1) transformed when necessary to

achieve normal distribution (Zar 1996). Because it was not possible to achieve normal distribution for shredder abundance data, Kruskal–Wallis tests were used, followed by Dunn's multiple comparison tests (Zar 1996).

Non-linear regressions were used to investigate the relationships between stream water variables, described by the first PCA axis (eutrophication gradient), and leaf mass loss, macroinvertebrate taxon richness, and shredder taxon richness. Linear regressions were used to investigate the relationships between nitrogen content in decomposing leaves (after stream immersion) and leaf mass loss.

Ordination by non-metric multidimensional scaling (NMDS), based on the Bray–Curtis similarity matrix after log(x+1) transformation, was used to analyze the distribution of leaf-associated macroinvertebrate assemblages by the streams and leaf species. Canonical Correspondence Analysis (CCA) was used to determine the relationships between stream water variables and mean abundance of leaf-associated invertebrate families (log(x+1) transformed, with down-weighting of rare species). Monte Carlo permutation tests based on 499 permutations were used to test the null hypothesis that leaf-associated invertebrate assemblages were unrelated with the environmental variables (Lepš and Šmilauer 2003).

The Iberian Biological Monitoring Working Party Index (IBMWP) and the Iberian Average Score Per Taxon (IASPT) were applied to benthic macroinvertebrates sampled with the hand net at each sampling stream (Alba-Tercedor 1996, Jáimez-Cuéller et al. 2002). A minimum of 2 individuals per taxon was the criterion to consider the presence of a taxon in a stream. The IBMWP index classifies water quality into five classes: I, very good water quality (IBMWP>100); II, good water quality (100≥IBMWP>61); III, moderate water quality (60≥IBMWP>36); IV, low water quality (35≥IBMWP>16); V, bad water quality (IBMWP≤15). The IASPT index was calculated as the average score per taxon, i.e. IBMWP/number of IBMWP families.

Several diversity measures were used: Shannon diversity index (H'), Pielou eveness index (E) (Legendre and Legendre 1998), invertebrate abundance, taxon richness, %EPT taxa, and %Oligochaeta taxa.

The relationships between biotic indices or diversity measures and the stream water variables, described by the first PCA axis or nutrient concentrations in stream water ($N\text{-}NH_4^+$, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$), were assessed by Spearman rank correlations.

PCA and CCA analyses were done with CANOCO 4.5 (Microcomputer Power, NY, USA). Shannon and Pielou indices and NMDS analyses were calculated with Primer v6 software package. The remaining statistical analyses were performed with Statistica 6.0 for Windows.

3. Results

3.1. Physical and chemical parameters of the stream water

Mean values of stream water temperature varied from 9.0 to 14.6ºC (Table 2.2). The lowest value was recorded in Agra Stream, which may be due to the high altitude of this site, while the highest value was recorded in Couros Stream. The latter stream also had the highest values for conductivity (324µs cm^{-1}), and concentration of $N\text{-}NH_4^+$ (3650µg L^{-1}), $P\text{-}PO_4^{3-}$ (272µg L^{-1}) and $N\text{-}NO_2^-$ (175µg L^{-1}). The highest concentrations of $N\text{-}NO_3^-$ were recorded in Agrela Stream (3367µg L^{-1}), Couros Stream (3200µg L^{-1}), and Selho River (3000µg L^{-1}), which can be due to the high population density, industrial and agricultural activities near the sampling sites. Dissolved oxygen in the stream water was lower in the Couros Stream (5.9mg L^{-1}), and similar among all the other streams. The PCA ordination of the stream water variables showed that the first PCA axis explained 98.3% of the variance, while the second axis explained 1.6% (Fig. 2.2). The variables indicative of eutrophication ($N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, $N\text{-}NH_4^+$ and conductivity) were negatively correlated with PC1. Streams were ordinated according to the eutrophication gradient as

follows: Agra Stream < Oliveira Stream < Andorinhas Stream < Agrela Stream < Selho River, Costa Stream < Couros Stream.

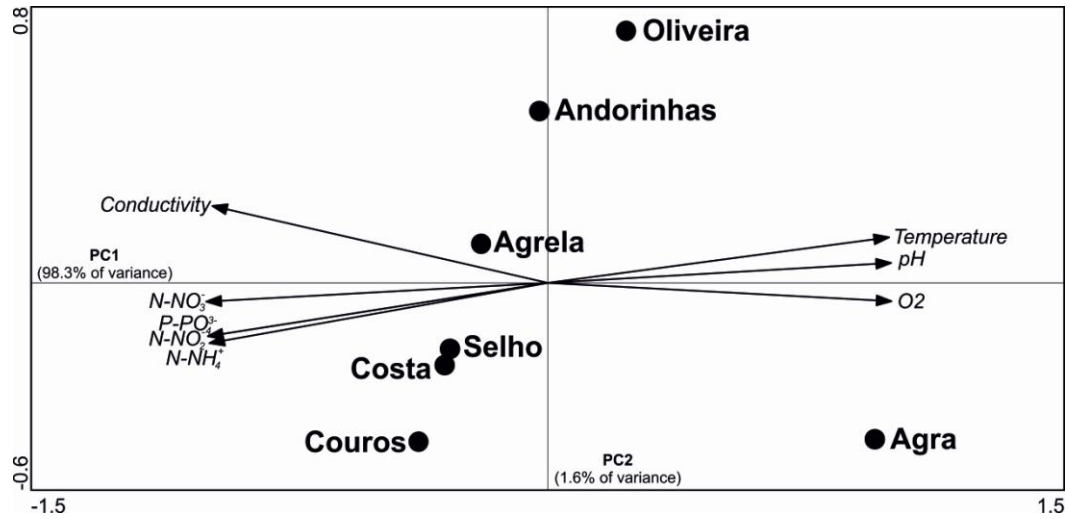

Figure 2.2. Principal component analysis (PCA) of the chemical and physical stream water variables at seven streams of the Ave River basin. The direction of the arrows reflects the maximum variation of each stream water variable.

3.2. Benthic macroinvertebrates and water quality

A total of 13406 benthic macroinvertebrates distributed among 80 families were sampled in the seven streams of the Ave River basin. Invertebrate abundance was higher in Selho River and lower in Costa Stream (Table 2.3). Taxon richness varied between 14 and 55 families in Selho River and Oliveira Stream, respectively. Streams with intermediate levels of eutrophication (Oliveira, Andorinhas and Agrela streams) had higher richness of invertebrates (Table 2.3). The percentage of EPT taxa had the highest values in Agra Stream, the less eutrophic stream, while the lowest values were found in Couros Stream. The percentage of Oligochaeta taxa was higher in Selho River and Couros Stream. Shannon index was higher in Andorinhas Stream (H'=3.1) and lower in Selho River and Couros Stream (H'=1.5). Pielou index was higher in Costa Stream, Andorinhas Stream and Oliveira Stream (J'=0.8) and lower in Agra Stream and Couros Stream

Table 2.2. Physical and chemical variables of the stream water at each sampling site of the Ave River basin during the study period. Data are mean values ± SEM (n = 1-5).

Variable	Agra Stream	Oliveira Stream	Andorinhas Stream	Agrela Stream	Selho River	Costa Stream	Couros Stream
Temperature (ºC)	9.0 ± 1.3	11.6 ± 1.3	12.0 ± 1.2	12.6 ± 0.6	12.9 ± 0.7	13.9 ± 1.1	14.6 ± 1.1
pH	5 ± 0.2	7 ± 0.1	7 ± 0.05	7 ± 0.2	7 ± 0.04	7	7 ± 0.1
Conductivity (µs cm^{-1})	16 ± 0.6	39 ± 0.9	59 ± 3	97 ± 3.6	154 ± 19.5	182 ± 14.0	324 ± 23.0
Dissolved O_2 (mg L^{-1})	11.2 ± 0.2	11.1 ± 0.3	10.2 ± 0.2	10.3 ± 0.1	10.0 ± 0.1	9.8 ± 0.2	5.9 ± 0.2
N-NO_2^- (µg L^{-1})	5.0 ± 1.0	5.0 ± 1.0	5.0 ± 1.0	5.5 ± 1.0	26.3 ± 9.0	26.0 ± 13.1	175.3 ± 5.8
N-NO_3^- (µg L^{-1})	160.0 ± 26.5	766.7 ± 109.3	1200.0 ± 173.2	3366.7 ± 145.3	3000.0	1900.0	3200.0
P-PO_4^{3-} (µg L^{-1})	2.2 ± 1.1	4.4 ± 1.1	4.4 ± 1.1	6.7 ± 3.3	56.7 ± 0.0	4.4 ± 1.1	272.2 ± 10.6
N-NH_4^+ (µg L^{-1})	10.0 ± 0.0	100.0	10.0 ± 0.0	30.0 ± 15.3	370.0 ± 87.4	340.0 ± 205.0	3650.0 ± 650.0

(J'=0.5). The IBMWP biotic index indicated that Agra, Oliveira, Andorinhas and Agrela streams had very good water quality (Class I), Costa Stream had good water quality (Class II), and Selho River and Couros Stream had low water quality (Class IV). IASPT values were higher in Agra, Oliveira, Andorinhas and Agrela streams than in Selho River, Costa and Couros streams (Table 2.3).

Table 2.3. Diversity measures and biotic indices applied to benthic macroinvertebrate community sampled with a hand net in seven streams of the Ave River basin.

Metric	Agra Stream	Oliveira Stream	Andorinhas Stream	Agrela Stream	Selho River	Costa Stream	Couros Stream
Abundance	794	2248	2198	2677	3625	336	1528
Taxon richness	30	55	54	47	14	31	18
% EPT taxa[a]	60.0	40.0	38.9	38.3	7.1	19.4	5.6
% Oligochaeta taxa	6.7	9.1	7.4	12.8	35.7	12.9	27.8
Shannon index, H'	1.7	3.0	3.1	2.4	1.5	2.7	1.5
Pielou index, E	0.5	0.8	0.8	0.6	0.6	0.8	0.5
IBMWP[b]	135	280	244	211	26	95	25
IASPT[c]	6.8	6.7	6.4	6.8	3.3	5.0	3.1

[a] EPT – Ephemeroptera, Plecoptera and Trichoptera; [b] IBMWP – Iberian Biological Monitoring Working Party Index; [c] IASPT - Iberian Average Score Per Taxon.

The IBMWP was negatively correlated with $N\text{-}NO_2^-$, while the IASPT was negatively correlated with $N\text{-}NH_4^+$ in the stream water (Spearman rank correlation, p=0.0073 and 0.0334, respectively). The EPT taxa (%) were negatively correlated with PC1, and specifically with the concentration of $P\text{-}PO_4^{3-}$, $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ in the stream water (Table 2.4). The Oligochaeta taxa (%) was positively correlated with all the above variables, except for $N\text{-}NO_3^-$.

3.3. Macroinvertebrates on decomposing leaves

A total of 68 families were recovered from leaf bags in the seven sampling sites along the Ave River basin. Shredders were represented by 13

Table 2.4. Spearman rank correlations between diversity measures or biotic indices applied to macroinvertebrates and the eutrophication gradient (as PC1 scores) or nutrients in the stream water.

Metric	PC1 score		Nutrient in the stream water							
			$N\text{-}NH_4^+$		$N\text{-}NO_2^-$		$N\text{-}NO_3^-$		$P\text{-}PO_4^{3-}$	
	r	p	r	p	r	p	r	p	r	p
Abundance	0.07	0.8790	0.11	0.8175	0.07	0.8745	0.39	0.3833	0.48	0.2736
Taxon richness	0.50	0.2532	-0.56	0.1925	-0.74	0.0566	-0.32	0.4821	-0.48	0.2736
% EPT taxa	**0.96**	**0.0005**	**-0.86**	**0.0120**	**-0.96**	**0.0005**	**-0.79**	**0.0362**	**-0.89**	**0.0073**
% Oligochaeta taxa	**-0.86**	**0.0137**	**0.92**	**0.0034**	**0.93**	**0.0027**	0.71	0.0713	**0.85**	**0.0148**
Shannon index, H'	0.39	0.3833	-0.58	0.1754	-0.70	0.0774	-0.39	0.3833	-0.56	0.1950
Pielou index, E	-0.14	0.7599	-0.09	0.8477	-0.19	0.6908	0.00	1.0000	-0.19	0.6908
IBMWP	0.75	0.0522	-0.72	0.0676	**-0.89**	**0.0073**	-0.46	0.2939	-0.59	0.1605
IASPT	0.75	0.0522	**-0.79**	**0.0334**	-0.74	0.0566	-0.25	0.5887	-0.59	0.1605

families, mainly Trichoptera. Andorinhas Stream had the highest taxon richness (47 families). The most common invertebrate taxa were Chironomidae and Lumbriculidae, and the most common shredders were Tipulidae, Leuctridae, Nemouridae, Limnephilidae, Lepidostomatidae and Sericostomatidae. Abundance and density of macroinvertebrates were higher in Selho River and lower in Costa Stream (Fig 2.3A, B). In Selho River, macroinvertebrate abundance ranged from 211 to 265 individuals/leaf bag in oak and chestnut leaves, respectively, and the assemblage was dominated by Oligochaeta and Chironomidae. Macroinvertebrate abundance in the Costa Stream ranged from 8 to 21 individuals/leaf bag in eucalyptus and chestnut leaves, respectively (Fig. 2.3A). Invertebrate abundance was significantly affected by eutrophication, but not by leaf identity or interaction between leaf identity and eutrophication (Two-way ANOVA, $p<0.05$; Table 2.5). Macroinvertebrate density ranged from 82 to 508 individuals/g dry mass (for plane tree and alder leaves, respectively) in Selho River, and from 2 to 8 individuals/g dry mass (for oak and chestnut leaves, respectively) in Costa Stream (Fig. 2.3B). Invertebrate density was significantly affected by leaf identity and eutrophication, but not by the interaction between factors (Two-way ANOVA, $p<0.05$; Table 2.5). Macroinvertebrate density was significantly

higher in alder leaves, followed by chestnut leaves, intermediate in plane tree and oak leaves and lower in eucalyptus leaves (Tukey's tests, $p<0.05$; Table 2.5).

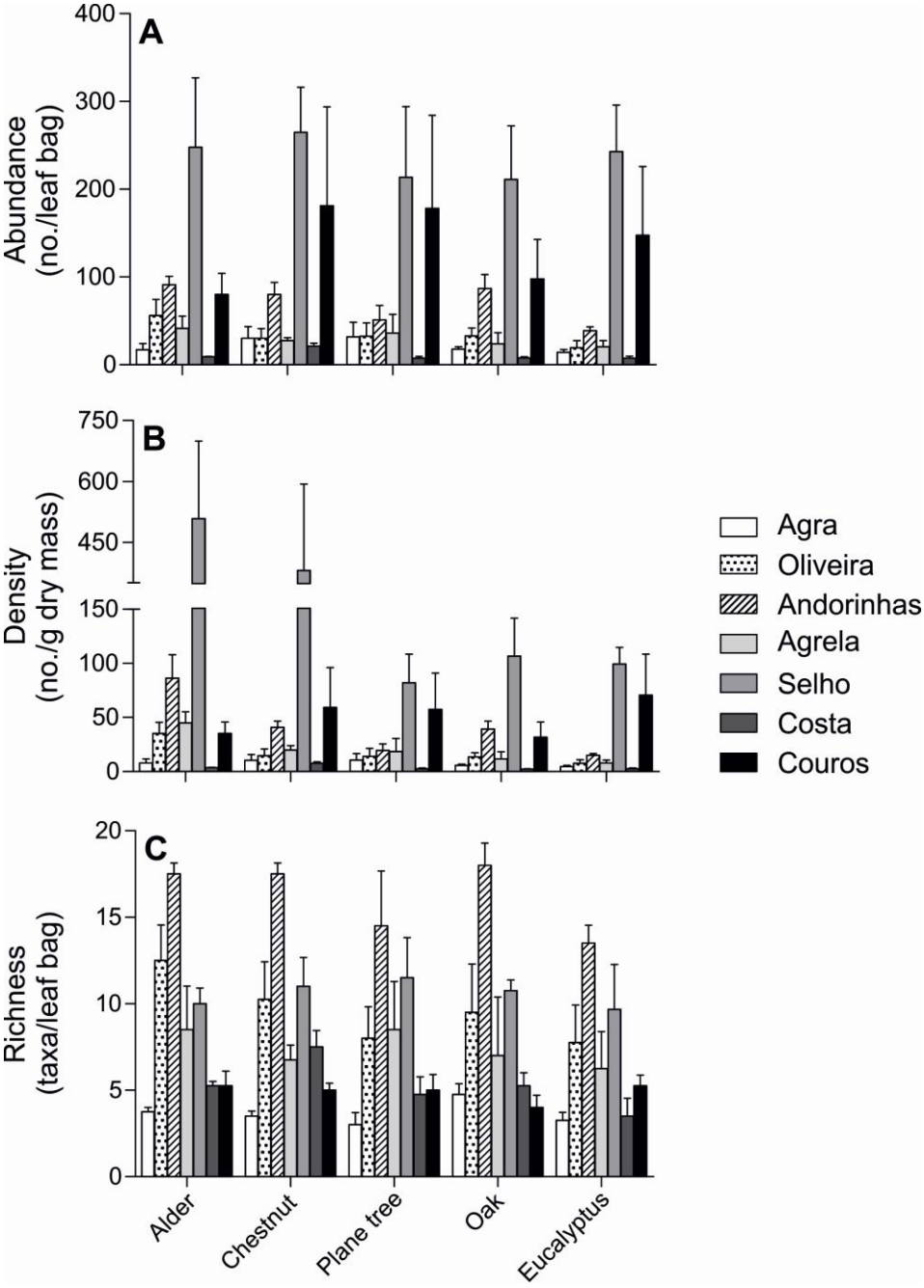

Figure 2.3. Macroinvertebrate abundance (A), density (B) and taxon richness (C) on leaves of alder, chestnut, plane tree, oak and eucalyptus immersed at seven sampling sites in the Ave River basin during 37 days. Streams are ordered according to the gradient of eutrophication defined by the PCA analysis. Mean + SEM; n=4.

Richness in invertebrate taxa ranged from 4 to 18 taxa/leaf bag in oak and from 3 to 14 taxa/leaf bag in eucalyptus leaves (Fig. 2.3C). Invertebrate taxon richness was significantly affected by eutrophication, but not by leaf identity and interactions between factors (Two-way ANOVA, $p<0.05$; Table 2.5). Generally, Oliveira, Andorinhas and Agrela streams and Selho River had significantly higher richness in invertebrate taxa than the other sites (Tukey's tests, $p<0.05$; Table 2.5).

Richness in shredder taxa ranged from 0 to 6 taxa/leaf bag for oak, and from 0.3 to 4 taxa/leaf bag for eucalyptus leaves (Fig. 2.4B). Shredder taxon richness was significantly affected by leaf identity and eutrophication, but not by the interaction between factors (Two-way ANOVA, $p<0.05$; Table 2.5). Shredder richness was significantly lower in eucalyptus than in chestnut leaves (Tukey's tests, $p<0.05$; Table 2.5).

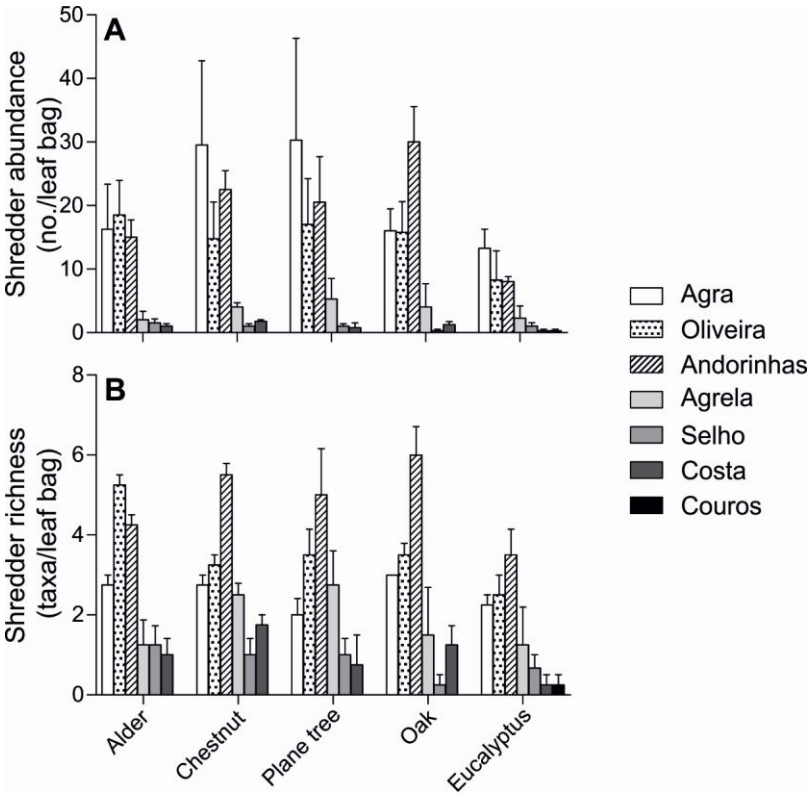

Figure 2.4. Shredder abundance (A) and taxon richness (B) on leaves of alder, chestnut, plane tree, oak and eucalyptus immersed at seven sampling sites in the Ave River basin during 37 days. Streams are ordered according to the gradient of eutrophication defined by the PCA analysis. Mean + SEM; n=4.

Generally, shredder abundance on leaves tended to decrease along the gradient of eutrophication for all leaf species (Fig. 2.4A), reaching higher values on plane tree (from 0 to 30 individuals/leaf bag), and lower on eucalyptus leaves (from 0.3 to 13 individuals /leaf bag). Shredder abundance was significantly affected by eutrophication, but not by leaf identity (Kruskal-Wallis test, $p<0.05$ and $p>0.05$, respectively). Shredder abundance was significantly higher in Agra, Oliveira and Andorinhas than in the other streams (Dunn's Test, $p<0.05$).

A non-linear regression analyses (second order polynomial model) confirmed that taxon richness of macroinvertebrates (Fig. 2.5A) and shredders (Fig. 2.5B) were generally higher at intermediate levels of eutrophication for all leaf species (Table 2.6).

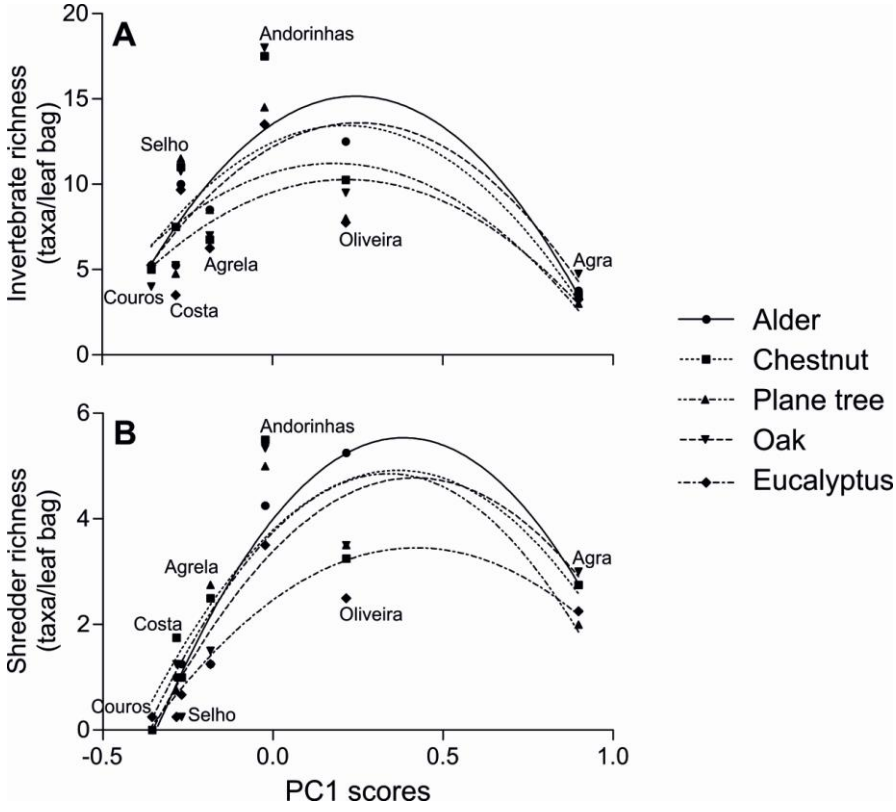

Figure 2.5. Relationships between taxon richness of macroinvertebrates (A) or shredders (B) on leaves of alder, chestnut, plane tree, oak and eucalyptus against the eutrophication gradient defined by PC1 based on the stream water variables at the sampling sites of the Ave River basin (Fig 2.2).

Table 2.5. Two-way ANOVA of the effects of leaf identity and eutrophication on macroinvertebrate abundance, macroinvertebrate density, macroinvertebrate richness, shredder richness, and leaf mass loss. Treatments with the same letter means no significantly differences ($p<0.05$) following Tukey HSD post-hoc testing.

Parameter	Effect	df	SS	MS	F	p	Tukey HSD post-test
Macroinvertebrate abundance	Leaf identity	4	1.064	0.266	1.591	0.18222	
	Eutrophication	6	21.527	3.588	21.452	<0.00001	Agra^{ad}, Oliveira^{ab}, Andorinhas^{b}, Agrela^{ad}, Selho^{c}, Costa^{d}, Couros^{b}
	Leaf identity*Eutrophication	24	1.332	0.056	0.332	0.99854	
	Error	104	17.394	0.167			
Macroinvertebrate density	Leaf identity	4	3.641	0.910	5.731	0.00033	Alder^{a}, Chestnut^{ab}, Plane tree^{bc}, Oak^{bc}, Eucalyptus^{c}
	Eutrophication	6	27.537	4.589	28.898	<0.00001	Agra^{ac}, Oliveira^{ad}, Andorinhas^{b}, Agrela^{ab}, Selho^{e}, Costa^{c}, Couros^{bd}
	Leaf identity*Eutrophication	24	2.532	0.106	0.664	0.87482	
	Error	104	16.517	0.159			
Macroinvertebrate richness	Leaf identity	4	67.342	16.835	1.624	0.17386	
	Eutrophication	6	2244.402	374.067	36.074	<0.00001	Agra^{a}, Oliveira^{bd}, Andorinhas^{c}, Agrela^{be}, Selho^{d}, Costa^{ae}, Couros^{ae}
	Leaf identity*Eutrophication	24	128.502	5.354	0.516	0.96756	
	Error	104	1078.417	10.369			
Shredder richness	Leaf identity	4	12.308	3.077	2.972	0.02277	Alder^{ab}, Chestnut^{a}, Plane tree^{ab}, Oak^{ab}, Eucalyptus^{b}
	Eutrophication	6	339.847	56.641	54.712	<0.00001	Agra^{a}, Oliveira^{b}, Andorinhas^{c}, Agrela^{ae}, Selho^{df}, Costa^{ef}, Couros^{df}
	Leaf identity*Eutrophication	24	37.761	1.573	1.520	0.07734	
	Error	104	107.667	1.035			
Leaf mass loss	Leaf identity	4	14521.9	3630.5	28.650	<0.00001	Alder^{a}, Chestnut^{b}, Plane tree^{b}, Oak^{b}, Eucalyptus^{b}
	Eutrophication	6	19109.7	3185.0	25.135	<0.00001	Agra^{a}, Oliveira^{b}, Andorinhas^{bc}, Agrela^{bc}, Selho^{c}, Costa^{a}, Couros^{a}
	Leaf identity*Eutrophication	24	6380.6	265.9	2.098	0.00548	
	Error	105	13305.2	126.7			

The NMDS ordination revealed that macroinvertebrate communities associated with leaves were structured by the stream more than by the leaf type (Fig 2.6). Macroinvertebrate communities discriminated four main groups, namely: (i) Agra Stream, (ii) Oliveira Stream, Andorinhas Stream, Agrela Stream, (iii) Costa Stream, and (iv) Selho River and Couros Stream.

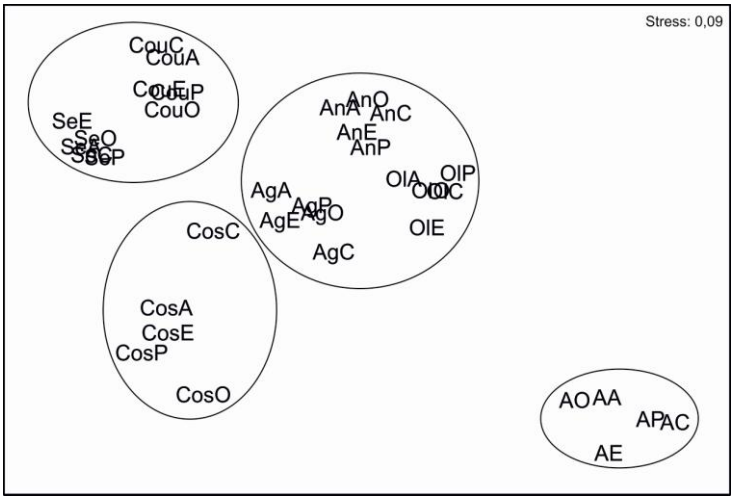

Figure 2.6. NMDS ordination of benthic invertebrate communities on leaves along the seven streams of the Ave River basin and according to the leaf type. AA, Agra Alder; AC, Agra Chestnut; AP, Agra Plane tree; AO, Agra Oak; AE, Agra Eucalyptus; OA, Oliveira Alder; OC, Oliveira Chestnut; OP, Oliveira Plane tree; OO, Oliveira Oak; OE, Oliveira Eucalyptus; AnA, Andorinhas Alder; AnC, Andorinhas Chestnut; AnP, Andorinhas Plane tree; AnO, Andorinhas Oak; AnE, Andorinhas Eucalyptus; AgA, Agrela Alder; AgC, Agrela Chestnut; AgP, Agrela Plane tree; AgO, Agrela Oak; AgE, Agrela Eucalyptus; SeA, Selho Alder; SeC, Selho Chestnut; SeP, Selho Plane tree; SeO, Selho Oak; SeE, Selho Eucalyptus; CosA, Costa Alder; CosC, Costa Chestnut; CosP, Costa Plane tree; CosO, Costa Oak; CosE, Costa Eucalyptus; CouA, Couros Alder; CouC, Couros Chestnut; CouP, Couros Plane tree; CouO, Couros Oak; CouE, Couros Eucalyptus.

CCA ordination shows the relationships between the stream water variables and the abundance of invertebrates associated with leaves in the seven sampling sites (Fig. 2.7). The first and the second axis explained 78% of the total variance attributed to environmental variables (Fig. 2.7). The structure of invertebrate communities and the stream water variables were

highly correlated with axis 1 and axis 2 (r=0.99 and r=0.95, respectively), indicating that the macroinvertebrate communities responded to differences in stream water variables. Monte Carlo permutation tests indicated that correlations were significant (p=0.002). All inorganic nutrients in the stream water ($N\text{-}NO_2^-$, $P\text{-}PO_4^{3-}$, $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$) and conductivity contributed to explain the structure of the invertebrate communities (p<0.05). Overall, environmental variables explained 59% of the variance.

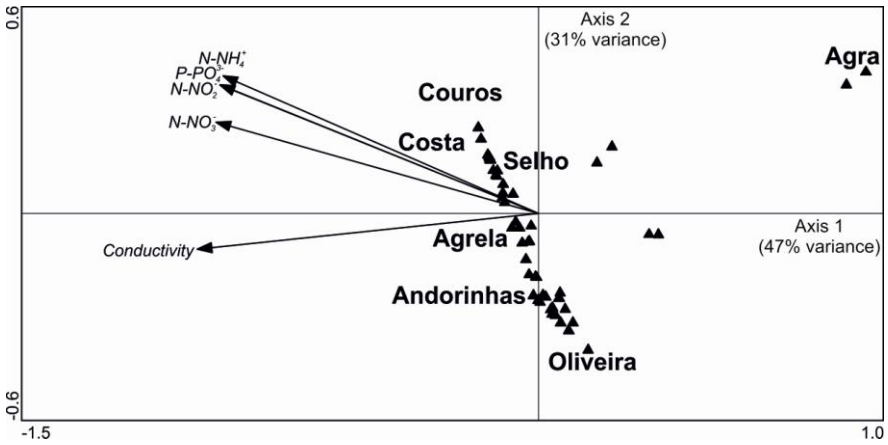

Figure 2.7. Canonical correspondence analysis (CCA) diagram for ordination of stream water variables indicative of eutrophication and invertebrate communities on decomposing leaves at the seven sampling sites. Biotic data represent invertebrate abundance of each taxon. The length of the arrows indicates the relative importance of each variable in explaining variation.

Table 2.6. Non-linear regressions (second order polynomial model) of invertebrate and shredder richness on leaves against the PC1 of the stream water variables after 37 days of leaf immersion.

	Invertebrate richness vs PC1 scores		**Shredder richness vs PC1 scores**	
Leaf type	r^2	p	r^2	p
Alder	0.58	<0.0001	0.84	<0.0001
Chestnut	0.46	0.0007	0.59	<0.0001
Plane tree	0.30	0.0132	0.51	0.0003
Oak	0.33	0.0080	0.57	<0.0001
Eucalyptus	0.30	0.0145	0.49	0.0004

3.4. Leaf decomposition

After 37 days of leaf immersion at the seven sampling sites of the Ave River basin, leaf mass loss attained maximum values for alder in the Selho River (84.98%) and minimum values for chestnut leaves in the Agra Stream (11.25%) (Fig. 2.8). Leaf mass loss was affected by plant identity, eutrophication and interaction between the two factors (two-way ANOVA, $p<0.05$; Table 2.5). Overall, leaf mass loss was significantly higher in streams with intermediate levels of eutrophication than in streams with low (Agra Stream) or high (Costa Stream and Couros Stream) levels of eutrophication (Tukey's tests, $p<0.05$; Table 2.5). Alder leaves decomposed significantly faster when compared with chestnut, plane tree, oak and eucalyptus leaves (Tukey's tests, $p<0.05$; Table 2.5).

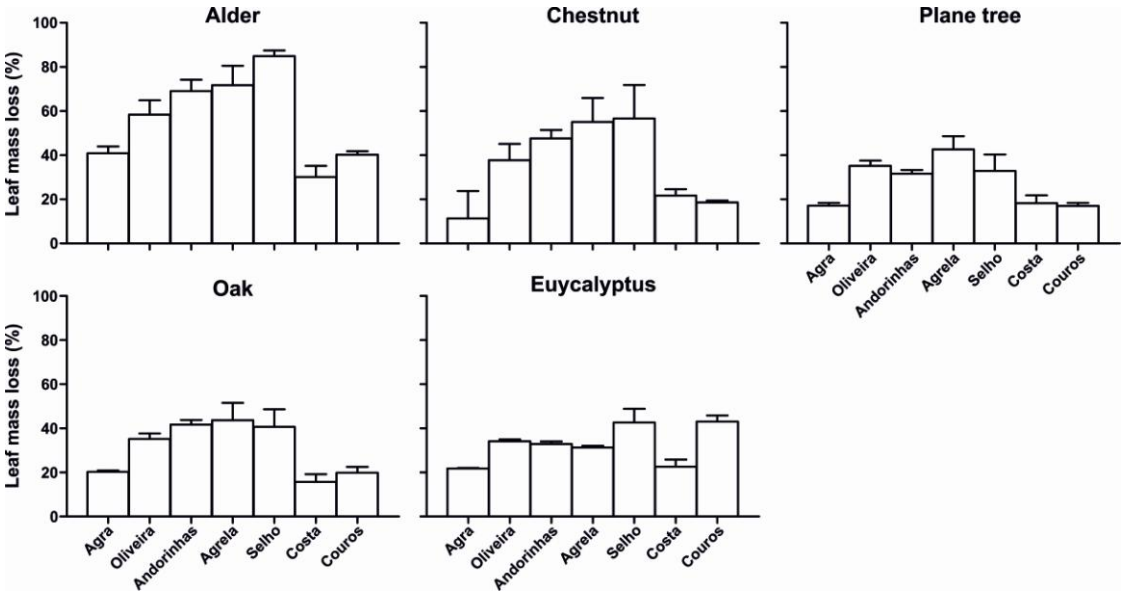

Figure 2.8. Leaf mass loss of alder, chestnut, plane tree, oak and eucalyptus leaves in coarse-mesh bags incubated in seven sampling sites of the Ave River basin, during 37 days. Streams were ordered according to the gradient of eutrophication defined by the PC1. Mean + SEM; n=4.

Non-linear regression analyses (second order polynomial model) applied to leaf mass loss along the gradient of eutrophication (first PCA scores) were significant and confirmed that leaf mass loss was stimulated by intermediate levels of eutrophication for chestnut (r^2=0.26, p=0.024), plane tree (r^2=0.34, p=0.005) and oak (r^2=0.29, p=0.015), but not for alder (r^2=0.19, p=0.068) leaves (Fig. 2.9). For eucalyptus leaves, leaf mass loss tended to increase linearly along the gradient of eutrophication (r^2=0.23, p=0.037; Fig. 2.9).

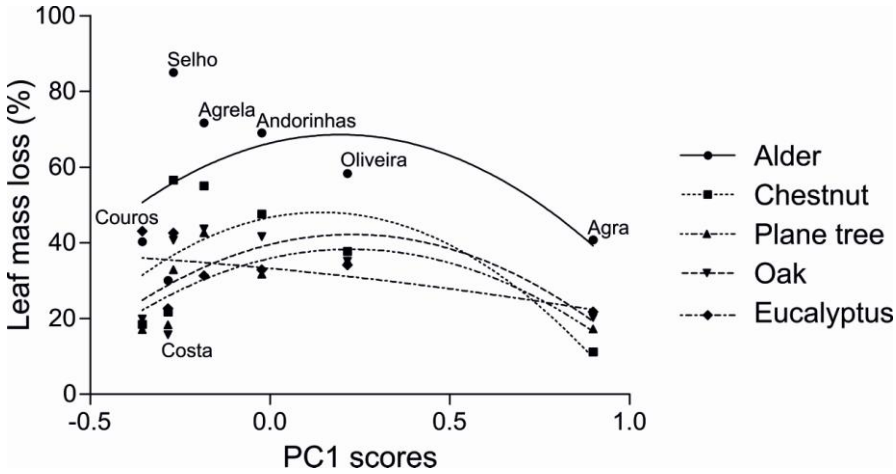

Figure 2.9. Regressions of leaf mass loss of alder, chestnut, plane tree, oak and eucalyptus against the eutrophication gradient defined by the first axis of PCA of the stream water variables in the sampling sites of the Ave River basin (Fig. 2.2).

Carbon content in leaves attained higher values for eucalyptus leaves in the Couros Stream (58.61%) and lower values for chestnut leaves in the Costa Stream (39.52%, Fig. 2.10). Nitrogen content in leaves immersed at the streams tended to increase for all leaf species when compared to their initial values (Fig. 2.11). The highest values of nitrogen content were found in alder leaves (from 4.32 to 5.15%) and lowest in eucalyptus leaves (from 2.02 to 3.01%, Fig. 2.11). Generally, nitrogen content was higher in alder leaves, followed by chestnut, oak, plane tree and eucalyptus leaves. Significant linear regressions were found between leaf mass loss and percentage of nitrogen on decomposing leaves for chestnut (r^2=0.40, p=0.0004), plane tree (r^2=0.47,

p<0.0001), oak (r^2=0.44, p=0.0001), and eucalyptus (r^2=0.19, p=0.0188) leaves.

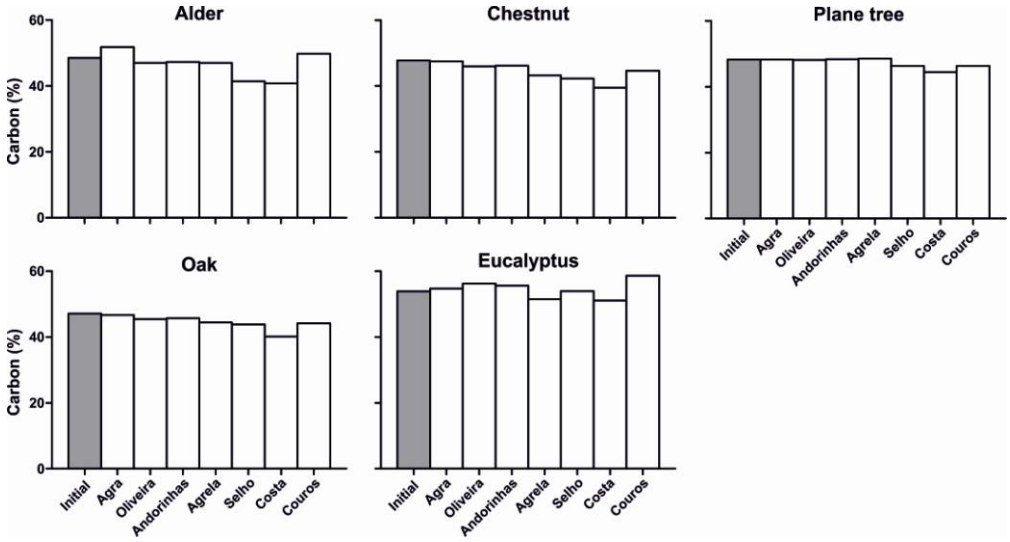

Figure 2.10. Percentage of carbon content of alder, chestnut, plane tree, oak and eucalyptus leaves after stream immersion. Initial values for carbon content are also given (gray bars). Streams are ordered according to the gradient of eutrophication defined by the axis 1 of PCA analysis.

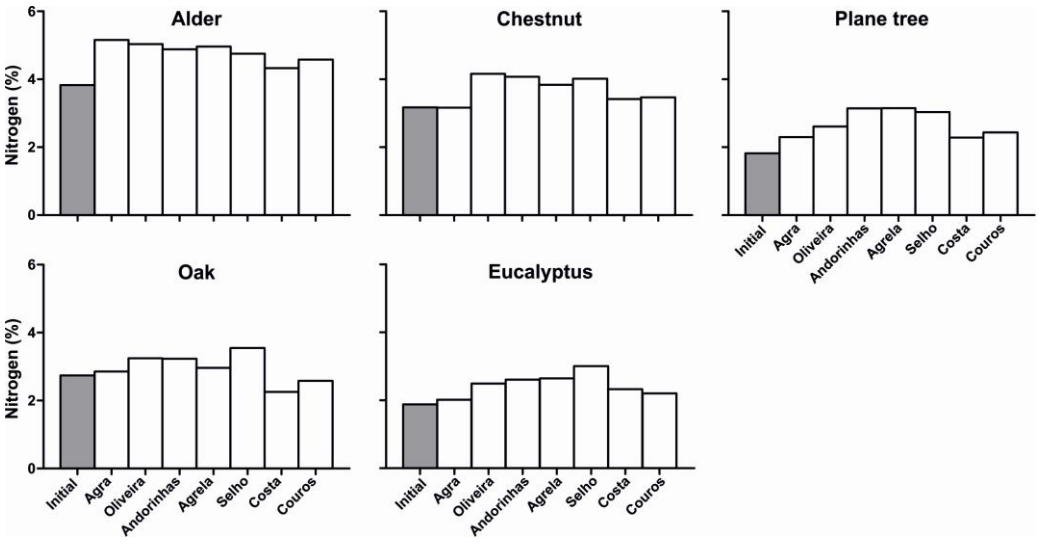

Figure 2.11. Percentage of nitrogen content of alder, chestnut, plane tree, oak and eucalyptus leaves after stream immersion. Initial values for nitrogen content are also given (gray bars). Streams are ordered according to the gradient of eutrophication defined by the axis 1 of PCA analysis.

4. General discussion and future perspectives

The ordination of streams according to the stream water parameters showed a separation of sites along an eutrophication gradient, being Agra Stream the less eutrophic site, followed by Oliveira Stream, Andorinhas Stream, Agrela Stream, Selho River, Costa Stream and Couros Stream. In the most eutrophic stream, the elevated concentrations of NO_2^-, NO_3^-, PO_4^{3-} and NH_4^+ could be related to the fact that this stream suffers the influence of the city of Guimarães and of the surrounding agricultural fields.

As expected, benthic macroinvertebrate communities were able to discriminate the degree of eutrophication of the studied streams. In fact, the richness of more sensitive taxa to eutrophication, such as Ephemeroptera, Plecoptera and Trichoptera, was higher in Agra, Oliveira, Andorinhas and Agrela streams, while that of tolerant taxa, such as Oligochaeta, was higher in Selho, Costa and Couros streams. The use of benthic invertebrate derived metrics, as indicators of environmental stress, has been widely used (e.g., Pascoal et al. 2001, 2003, Castela et al. 2008). The application of the IBMWP biotic index indicated that Agra, Oliveira, Andorinhas and Agrela streams had very good water quality and Selho River and Couros Stream had low water quality. The IASPT index was also able of discriminate Agra, Oliveira, Andorinhas and Agrela streams from all the other streams, confirming that the less eutrophic sites had higher sensitive taxa than the most eutrophic sites.

Leaf-associated macroinvertebrate community also shifted along the gradient of eutrophication, as indicated by the NMDS ordination of the invertebrates associated with leaf litter. Abundance and density of leaf-associated invertebrates tended to increase in the most eutrophic streams, which is in agreement with other studies reported in literature (Robinson and Gessner 2000, Pascoal et al. 2001, 2003, Niyogi et al. 2003, Pascoal et al. 2005, Gulis et al. 2006, Bergfur et al. 2007b). However, invertebrate taxon richness and shredder taxon richness increased until intermediate levels of eutrophication and decreased in the most eutrophic sites, while abundance of

invertebrate shredders decreased along the gradient of eutrophication. This may be attributed to the sensitivity of several invertebrate groups to eutrophication (Pascoal et al. 2001, 2003, Bergfur et al. 2007b), including shredders (Pascoal et al. 2001, 2003, Baldy et al. 2007).

Some studies have reported that high levels of nutrients in stream water, mainly phosphorus and/or nitrate, stimulate the decomposition of leaves (Robinson and Gessner 2000, Gulis and Suberkropp 2003b, Pascoal et al. 2003). In the present study, decomposition of alder, chestnut, oak and plane tree leaves was stimulated by intermediate levels of eutrophication, but decreased in the most eutrophic streams. In fact, a hump-shaped relationship was found between leaf mass loss and the pollution gradient defined by the stream water variables, suggesting that leaf mass loss can be inhibited in highly eutrophic streams, as found by others (Gulis et al. 2006, Lecerf et al. 2006, Mesquita et al. 2007). This may be attributed to the occurrence of multiple stressors, including toxicants, which can decrease shredder abundance and retard leaf mass loss. For instance, Lecerf et al. (2006) found that breakdown rates of alder leaves decreased along a gradient of eutrophication defined by ammonium concentration in the stream water, probably due to the toxic effects of ammonia to benthic invertebrates. In our study, the most eutrophic site (Couros Stream) had i) high levels of nitrate and the highest levels of ammonium, phosphate and nitrite in the stream water, and ii) the retrieved bags were filed with fine sediments, with leaves showing a dark color. The latter aspect and the low oxygen concentration and current velocity in the Couros Stream suggest the presence of hypoxic conditions that could have contributed to the decline of shredders and inhibition of leaf-litter decomposition at this site. Previous studies in eutrophic streams of Northwest Portugal also reported slower decomposition rates at sites with high sedimentation and low current velocity (Pascoal and Cássio 2004, Pascoal et al. 2005a, Mesquita et al. 2007). In fact, it has been reported that high sedimentation contributes to the decline of benthic invertebrates

(Niyogi et al. 2003), and shredder abundance and density (Hagen et al. 2006) in streams.

In Costa Stream, although the concentrations of nutrients in stream water were not as high as in the Couros Stream, leaf decomposition was also inhibited. At this stream site, leaves had a dark color and an orange precipitate at the surface, which was also present on the stream bed and on macroinvertebrates associated with leaf bags. Unfortunately, we did not measure metals in the stream water or leaf litter. However, it is probable that the decrease of benthic invertebrates, including shredders at this site, may be the result of possible metal ions, including ferric iron, in the stream water, which resulted in the inhibition of leaf mass loss. In streams affected by mine drainage, an increased concentration of zinc and consequent deposition of metal oxides were related to a decrease in shredder biomass and rates of litter breakdown (Niyogi et al. 2001). On the other hand, in Agra Stream (less eutrophic), the slower leaf mass loss could be attributed to i) limiting nutrient concentration in the stream water, or ii) low temperature during the study period with possible occasional freezing, that could have inhibited the activity of aquatic biota, and consequently decelerated leaf mass loss. Fernandes et al. (2009) found that the freezing can retard leaf mass loss, and constrain the diversity and the ecological functions of aquatic fungi on decomposing leaves. This may have direct and/or indirect effects on benthic macroinvertebrates, mainly to shredders that feed on conditioned leaf litter (Bärlocher and Kendrick 1973, Arsuffi and Suberkropp 1985, Graça et al. 1993, Graça et al. 2001a, Graça and Cressa 2010, Lecerf et al. 2005).

In this study, invertebrate shredders could have been responsible for litter breakdown in streams with low and intermediate levels of eutrophication. However, at highly-eutrophic sites shredders were rare or even absente, suggesting that they had a minor role in leaf decomposition. Other invertebrates can opportunistically behave as shredders (Graça 2001, Pascoal et al. 2003, Mesquita et al. 2007). For instance, in Selho River, the fast leaf mass loss might be attributed not only to the physical fragmentation

of leaves due to the high current velocity, but also to the biotic fragmentation: a high number of oligochaetes was found inside the leaf matrix and several chironomids were associated with the leaf bags. Although oligochaetes are known to feed mainly on fine particulate organic matter and to use leaves as refugee, it is possible that leaf decomposition can be enhanced by their movements and feeding activity (Chauvet et al. 1993, Pascoal et al. 2003). Canhoto and Graça (1999) observed that, occasionally, chironomids feed on decomposing leaves of eucalyptus. This fact, could also contributed to the high leaf mass loss of eucalyptus leaves in Couros Stream. Furthermore, elevated concentration of nutrients, mainly nitrogen and phosphorus, in stream water enhance the activity of fungi and bacteria on decomposing leaves (Gulis and Suberkropp 2003a, b, Pascoal and Cássio 2004). Although we did not measure microbial functional parameters, it is conceivable that fungi and bacteria had increased their contribution to leaf litter decomposition in eutrophic streams, as suggested by others (Pascoal and Cássio 2004, Pascoal et al 2005a).

It has been shown that leaves with different nutritional quality decompose at different rates (Canhoto e Graça 1996, Sampaio et al. 2001, Abelho et al. 2008, 2009). For instance, leaves with high nitrogen concentration (e.g., alder) are reported to have fast decomposition rates (Pozo et al. 1998, Sampaio et al. 2001, Abelho et al. 2008, 2009). In our study, alder leaves decomposed significantly faster followed by chestnut, oak, plane tree and eucalyptus leaves. Additionally, it has been reported that shredders tend to be more abundant in leaves with high quality (high nitrogen and phosphorus concentrations) than in lower quality leaves (e.g., Webster and Benfield 1986, Irons et al. 1988, Pozo et al. 1998, Lecerf et al. 2005), explaning the increase in leaf mass loss. We observed that shredder richness was significantly higher in chestnut leaves than in low quality eucalyptus leaves. Unfortunately, we did not follow leaf colonization dynamic over time, and the probable earlier colonization of alder leaves by shredders could have been missed. Invertebrates in leaf bags might be looking for a shelter or

exploring food resources (Abelho 2008). On the other hand, the higher density of invertebrates on alder and chestnut leaves can be attributed to the higher nitrogen content in these leaf species. In low quality leaves, the presence of recalcitrant or inhibitory compounds could also be responsible for slower mass loss of certain leaf species, such as eucalyptus (Pozo et al. 1998, Canhoto e Graça 1996). For instance, it has been shown that certain compounds, such as oils and polyphenolics, and the presence of waxes in the surface of leaves can delay fungal colonization of eucalyptus leaves (Bärlocher et al. 1995, Canhoto and Graça 1999), and influence its consumption by shredders (Canhoto and Graça 1999).

Changes in the nitrogen content on decomposing leaves might be attributed to nutrient immobilization due to microbial colonization and growth on leaves, especially in waters with high nitrogen content (e.g., Chauvet et al. 1987, Sampaio et al. 2001, Gulis and Suberkropp 2003b, Ferreira et al. 2006). In this study, the higher concentrations of nitrogen in stream water at the most eutrophic sites did not result in a greater increase of the nitrogen content in decomposing leaves. This result is in agreement with those reported by Gulis et al. (2006), who did not find an increase in leaf nitrogen content in eutrophic streams.

Currently, most of the assessment programs of freshwater ecosystems are based on the analysis of chemical and physical parameters of the stream water, and on the structural aspects of aquatic biota (Barbour et al. 1999, EU 2000). However, it has been suggested that the assessment of ecological integrity of an ecosystem requires that both structural and functional measures are considered (Pascoal et al. 2001, Gessner and Chauvet 2002, Pascoal et al. 2003). Some authors have suggested the use of leaf-litter decomposition as an indicator of stream functional integrity, because leaf breakdown responds to stream water chemistry (Webster and Benfield 1986, Gessner and Chauvet 2002, Pascoal et al. 2001, 2003). In this study, stream functional integrity was evaluated based on leaf mass loss of five leaf species, and the structural integrity was assessed using biotic indexes and

other diversity metrics based on benthic macroinvertebrates. We observed that leaf mass loss changed along the gradient of eutrophication lending support to the idea that leaf-litter decomposition could be used as a complementary measure to assess stream ecosystem condition. Most decomposition studies have used alder leaves (e.g., Pascoal et al. 2001, 2003, Lecerf et al. 2006, Bergfur et al. 2007a), but recently it was proposed the use of slowly decomposing, tough and low-nutrient substrates, such as oak leaves (Gulis et al. 2006). In this study, alder, chestnut, oak and plane tree leaves responded better to water quality degradation than eucalyptus leaves. However, alder leaves seemed to give a faster response than the other leaf species, since it decomposed significantly faster.

Overall, the structure of benthic macroinvertebrate communities was sensitive to the water quality degradation and was able to discriminate the less eutrophic from the most eutrophic streams. Shredders were clearly not responsible for the decomposition at the most eutrophic sites. Leaf litter decomposition was stimulated until intermediate levels of eutrophication and decreased in the most eutrophic streams, where the concentration of nutrients and the possible co-occurrence of other pollutants could have inhibited decomposition. Leaf mass loss of alder leaves was significantly faster when compared to other leaf species, and macroinvertebrate community showed evidence of preferential colonization of high quality leaves.

In conclusion, leaf decomposition showed to be a promising tool to assess changes in stream water quality. In addition, comparing to macroinvertebrate community analysis, estimation of leaf mass loss has advantages associated with costs, time consume and simplicity of application. Overall, results from this study showed that both structural and functional measures were good predictors of eutrophication in streams. Therefore, these measures complemented each other and make an integrative assessment of the ecological condition of streams possible.

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